Arabinan-deficient mutants of Corynebacterium glutamicum and the consequent flux in decaprenylmonophosphoryl-D-arabinose metabolism


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The arabinogalactan (AG) of Corynebacte rianeae is a critical macromolecule that tethers mycolic acids to peptidoglycan, thus forming a highly impermeable cell wall matrix termed the mycolyl-arabinogalactan peptidoglycan complex (mAGP). The front line anti-tuberculosis drug, ethambutol (Emb), targets the Mycobacterium tuberculosis and Corynebacterium glutamicum arabinofuranosyltransferase Mt-EmbA, Mt-EmbB and Cg-Emb enzymes, respectively, which are responsible for the biosynthesis of the arabinan domain of AG. The substrate utilized by these important glycosyltransferases, decaprenylmonophosphoryl-D-arabinose (DPA), is synthesized via a decaprenylphosphoryl-5-phosphoribosyl (DPPR) synthase (UbiA), which catalyzes the transfer of 5-phospho-ribofuranose-pyrophosphate (pRpp) to decaprenol phosphate to form DPPR. Glycosyl compositional analysis of cell walls extracted from a C. glutamicum::ubiA mutant revealed a galactan core consisting of alternating β(1→5)-Gal and β(1→6)-Gal residues, completely devoid of arabinan and a concomitant loss of cell-wall-bound mycolic acids. In addition, in vitro assays demonstrated a complete loss of arabinofuranosyltransferase activity and DPA biosynthesis in the C. glutamicum::ubiA mutant when supplemented with p[14C]Rpp, the precursor of DPA. Interestingly, in vitro arabinofuranosyltransferase activity was restored in the C. glutamicum::ubiA mutant when supplemented with exogenous DPP[14C]A substrate, and C. glutamicum strains deficient in ubiA, emb, and aftA all exhibited different levels of DPA biosynthesis.

Key words: arabinogalactan/Corynebacterium glutamicum/ decaprenylmonophosphoryl-D-arabinose/mutants/ Mycobacterium tuberculosis

Introduction

Corynebacterianae, which include the human pathogens Corynebacterium diphtheriae and Mycobacterium tuberculosis, represent an extremely important group of Actinomycetales (Coyle and Lipsky, 1990; Bloom and Murray, 1992; Funke et al., 1997). They belong to the same suborder and share a similar genome, cell wall ultrastructure, and corresponding cell wall biosynthetic machinery (Dover et al., 2004). The cell envelope of these bacteria consist of a basal peptidoglycan, which is coupled to a lipid-rich mycolic acid layer by a heterogeneous polysaccharide, arabinogalactan (AG) (Daffe et al., 1990; McNeil et al., 1990, 1991; Besra et al., 1995; Dover et al., 2004). AG consists of alternating β(1→5) and β(1→6) linked galactofuranose residues and is polymerized by the bifunctional galactofuranosyltransferase, GltT (Mikusova et al., 2000; Kremer et al., 2001), which uses UDP-Gal as a high-energy sugar donor (Weston et al., 1997; Sanders et al., 2001), to produce a linear galactan domain. Three branched arabinan motifs are attached to the C5 position of a β(1→6) linked galactose (Gal) residue at the 8th, 10th, and 12th Gal residues of the galactan domain (Alderwick et al., 2005). The arabinan motifs consist of a linear α(1→5) arabinan core with branching introduced at specific C3 positions along the arabinan polysaccharide (Daffe et al., 1990; Besra et al., 1995; Alderwick et al., 2005). The AG terminates with β(1→2) linked Ara/units in a unique hexa-arabinofuranosyl motif, which is the site of mycolation (McNeil et al., 1991). The final stages of AG biosynthesis are unknown, but at some point, the entire mAG is ligated to the peptidoglycan (Hancock et al., 2002; Yagi et al., 2003). AG has been shown to be an essential macromolecule in Corynebacterianeae, such as M. tuberculosis (Pan et al., 2001; Mills et al., 2004). However, the generation of viable arabinan-deficient mutants of Corynebacterium glutamicum has proven to be inherently useful in the study of “essential” genes involved in mycobacterial cell wall biosynthesis (Alderwick et al., 2005, 2006).

Mycobacterium tuberculosis utilizes the genes encoded by aftA, embA, and embB to perform arabinan polymerization in AG (Belanger et al., 1996; Escuyer et al., 2001; Alderwick et al., 2005, 2006) and by embC in lipoarabinomannan (Zhang et al., 2003). In contrast, C. glutamicum possesses only one ethambutol (Emb) paralog (Alderwick et al., 2005), which is the sole enzyme used in AG biosynthesis. However, both M. tuberculosis and C. glutamicum utilize the lipid-linked sugar donor decaprenylmonophosphoryl-D-arabinose (DPA) as its high-energy sugar donor (Wolucka et al., 1994; Lee et al., 1995, 1998). DPA is initially synthesized via a pentose shunt pathway (Scherman et al., 1996), where 5-phospho-ribofuranose-pyrophosphate (pRpp) is transferred to decaprenylmonophosphate forming decaprenylphosphoryl-5-phosphoribose (DPPR) by the decaprenyl transferase UbiA (Huang et al., 2005) (Rv3806c and NCglI2789 in M. tuberculosis and C. glutamicum, respectively).
were aligned using ClustalW and epimerization at the C2 hydroxyl forming DPA (Mikusova et al., 2005). Herein, we show that a ubiA-disrupted mutant of C. glutamicum produces an arabinan-deficient cell wall which consists of a core linear galactan polymer, with the loss of arabinan and cell wall esterified corynomycolic acids. Furthermore, biochemical analysis of membranes isolated from the C. glutamicum::ubiA mutant was unable to synthesize DPA from exogenous pRpp. Interestingly, membranes isolated from this mutant exhibited unaffected arabinofuranosyltransferase activity indicating that the enzymes responsible for arabinan precursor and glycosyltransferase activity work independently. Taken together, this data suggest the essentiality of UbiA to synthesize DPA in Corynebacterianeae and expose a new drug target, which could possibly be a site of interest for future anti-mycobacterial drug development.

**Results**

**Disruption of Cg-ubiA**

The ubiA gene product was shown in prior work to synthesize DPR, which is converted to DPA, thus supplying the substrate for the “priming” arabinosyltransferase AtfA (Alderwick et al., 2006). We inactivated the mycobacterial ortholog of C. glutamicum, NCgl2781, by transforming the wild type to kanamycin resistance conferred by the vector pCg::aph. The vector was integrated into the chromosomal ubiA gene, thus disrupting ubiA, as confirmed by two independent polymerase chain reaction (PCR) analyses with two different primer pairs (data not shown). As expected, the resulting strain C. glutamicum::ubiA exhibited a strong reduced growth, as shown in Figure 1. It thus resembles the aftA inactivation mutant of C. glutamicum exhibiting an almost identical growth behavior (Alderwick et al., 2006).

**UbiA sequence analysis**

UbiA sequences from M. tuberculosis, Mycobacterium bovis, Mycobacterium avium paratuberculosis, and C. glutamicum respectively). DPR then undergoes dephosphorylation and epimerization at the C2 hydroxyl forming DPA (Mikusova et al., 2005). Herein, we show that a ubiA-disrupted mutant of C. glutamicum produces an arabinan-deficient cell wall which consists of a core linear galactan polymer, with the loss of arabinan and cell wall esterified corynomycolic acids. Furthermore, biochemical analysis of membranes isolated from the C. glutamicum::ubiA mutant was unable to synthesize DPA from exogenous pRpp. Interestingly, membranes isolated from this mutant exhibited unaffected arabinofuranosyltransferase activity indicating that the enzymes responsible for arabinan precursor and glycosyltransferase activity work independently. Taken together, this data suggest the essentiality of UbiA to synthesize DPA in Corynebacterianeae and expose a new drug target, which could possibly be a site of interest for future anti-mycobacterial drug development.

**Mycolyl-arabinogalactan composition**

C. glutamicum::ubiA

We have previously reported on the generation of C. glutamicum mutants that are truncated in their arabinan domains of AG or are completely arabinan deficient (Alderwick et al., 2005, 2006). This study highlights the generation of C. glutamicum mutants as an aid to probe underlying cell wall biosynthetic enzymes within Corynebacterianeae, which are predicted to be essential (Alderwick et al., 2005). Glycosyl linkage analysis of cell walls prepared from a C. glutamicum::ubiA mutant indicates that this mutant is completely devoid of an arabinan domain, whereas the galactan core remains unaffected compared with C. glutamicum (Figure 3), thus further corroborating our previous findings (Alderwick et al., 2005). The loss of mycolic acid esterification sites was also confirmed by analysis of cell-wall-bound corynomycolic acids, which proved to be absent in C. glutamicum::ubiA (Figure 4).

**Identification of cell-wall-associated and exported lipids**

Because the disruption of arabinan in C. glutamicum essentially results in a complete loss of the “upper” cell wall mycolyl-arabinogalactan peptidoglycan (mAGP) complex, we investigated the production and profile of cell-wall-associated and exported lipids in C. glutamicum and C. glutamicum arabinan-deficient mutants. Analysis of cell-wall-associated lipids, such as trehalose dicorynomycolates (TDCM), trehalose monocorynomycolates (TMCM), and phospholipids from C. glutamicum, in comparison with C. glutamicum::ubiA, C. glutamicum::aftA, and C. glutamicum::aftA suggested that C. glutamicum possessed a higher degree of cell-wall-associated lipids (Figure 6). It was interesting to note the increased level of free TMCM in comparison with TDCM and phospholipids in C. glutamicum::ubiA, C. glutamicum::aftA, and C. glutamicum::aftA compared with C. glutamicum. This suggests that perhaps those lipids, which would otherwise be present in larger quantities, were emancipated due to the loss of cell-wall-bound corynomycolic acids and arabinan, thus removing their intercalation with the mAGP complex. Metabolic labeling
Arabinan-deficient mutants of *Corynebacterium glutamicum* using [14C]acetate of growing cultures of *C. glutamicum*, *C. glutamicum::ubiA*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA* and analysis of spent culture filtrates revealed a dramatic increase in “exported” TDCM, TMCM, and phospholipids from the *C. glutamicum* mutants compared with *C. glutamicum* (Figure 5B).

**Endogenous arabinofuranosyltransferase activity of *C. glutamicum*, *C. glutamicum::ubiA*, and *C. glutamicumΔemb***

We assessed the capacity of membrane preparations from *C. glutamicum*, *C. glutamicum::ubiA*, and *C. glutamicumΔemb* to elicit arabinofuranosyltransferase activity in the presence of an endogenous cell wall acceptor. Analysis of membranes along with cell wall material extracted from *C. glutamicum*, and exogenous radiolabeled p[14C]Rpp, indicated severely reduced activity from *C. glutamicumΔemb* to turn over p[14C]Rpp via DP[14C]A and incorporation into cell wall polymer compared with *C. glutamicum* (Figure 6A). This was not surprising, because Emb is responsible for the majority of cell wall arabinan biosynthesis. However, the level of arabinan incorporation was not completely diminished due to the activity of Cg-AftA (Alderwick et al., 2006), which is able to transfer single arabinofuranosyl residues to the galactan core from DPA. Conversely, we could not observe any glycosyltransferase activity using membranes prepared from *C. glutamicum::ubiA* (Figure 6A). However, restoration of Emb activity could be observed in membranes prepared from *C. glutamicum::ubiA* when, rather than providing p[14C]Rpp as a substrate, exogenous DP[14C]A was included in assays (Figure 6B). This suggests that both Emb and UbiA work in a mutually exclusive manner and also indicates that Emb is unaffected by Cg-ubiA disruption. We could observe a slight increase in arabinofuranosyltransferase activity in membranes produced from *C. glutamicumΔemb* (Figure 6B), this however could be attributed to a higher level of DP[14C]A in the assay components, compared with the assay containing p[14C]Rpp which would require further endogenous metabolism into DP[14C]A.

**UbiA activity in membranes prepared from *C. glutamicum*, *C. glutamicum::ubiA*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA***

It has been previously reported (Huang et al., 2005) that UbiA is a 5-phospho-α-D-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase. We assayed membranes prepared from *C. glutamicum* and from various mutants described in this report for DP[14C]A biosynthetic activity. Membranes prepared from *C. glutamicum* were able to produce radiolabeled products that migrate on thin layer chromatography (TLC) corresponding to DPA (Lee et al., 1995, 1998; Scherman et al., 1996) and DPPR (Huang et al., 2005) standards (Figure 7A). This result was also observed with membranes prepared from *C. glutamicumΔemb* and *C. glutamicumΔaftA*, but with a highly significant increase in band density corresponding to DPP[14C]R and DP[14C]A/DP[14C]R in *C. glutamicumΔemb* and
Corynebacterium glutamicumΔaft strains, respectively. As expected, we could not observe formation of DPP[14C]R or DP[14C]A/DP[14C]R in membrane preparations from C. glutamicum::ubiA (Figure 7A). To confirm the sugar content of each individual product in these reactions, we extracted the bands by preparative TLC and analyzed the product hydrolysates in terms of sugar content (Figure 7B). Extraction and sugar analysis of the band corresponding to DPPR from C. glutamicum (Figure 7B, lane 1) indicated that this product contained exclusively ribose. Analysis of the band equivalent to DPA/decapenylmonophosphoryl-D-ribose (DPR) from C. glutamicum (Figure 7B, lane 2) indicated that there was a heterogeneous mix of ribose and arabinose (Ara), corresponding to a mixture of DPR and DPA, respectively. Interestingly, sugar analysis of products migrating to the positions of DPPR and DPA/DPR from C. glutamicumΔemb and C. glutamicumΔaftA gave different band density profiles for ribose and Ara (Figure 7B, lanes 4–6). It has not escaped our notice that the utilization of membranes prepared from C. glutamicumΔaftA would be a useful tool for the enzymatic synthesis of DPA (Figure 7A and B, lane 6). As a control, we excised bands in the regions which related to DPPR and DPA/DPR from C. glutamicum::ubiA, and, as expected, no sugars were observed (Figure 7B, lanes 7 and 8).
Discussion

Understanding the biosynthetic pathways involved in mycobacterial arabinan biosynthesis is paramount to identifying potential new drug targets for the treatment of tuberculosis. We have shown recently that gene deletion in *C. glutamicum* provides a useful tool in the understanding of mycobacterial cell wall biosynthesis, because deletion of orthologous genes in mycobacterial species often causes lethality (Gande et al., 2004; Alderwick et al., 2005, 2006).

More specifically, our earlier studies (Alderwick et al., 2005) examined the potential disruption of *ubiA* in *C. glutamicum* and its consequence at a whole cell level due to the observation that the singular Ara residues in the *emb*-deleted strain could possibly arise from another sugar donor, that is, we attempted to disrupt arabinan biosynthesis completely and examine the final incorporation of Ara into the cell wall at a whole cell level. Analysis of alditol acetates prepared from purified cell walls from the *Cg::ubiA* mutant established DPA as the sole donor of Ara residues in *C. glutamicum*. In this present report, we have now more carefully analyzed the *in vitro* biochemical phenotype of the *Cg::ubiA* mutant in terms of cell-wall-associated TDCM and TMCM glycolipids, “exported” glycolipids and phospholipids, *in vitro* arabinofuranosyltransferase activity of *C. glutamicum*, *C. glutamicum::ubiA*, and *C. glutamicum::emb* mutant in terms cell wall arabinan polymerization, and UbiA activity in membranes prepared from *C. glutamicum*, *C. glutamicum::ubiA*, *C. glutamicum::emb*, and *C. glutamicum::aftA* in terms of the biosynthesis of DPPR and DPA. These *in vitro* biochemical experiments were not described in our earlier studies (Alderwick et al., 2005) and provide a more comprehensive analysis of arabinan biosynthesis in our panel of mutants as discussed below. Huang and others (2005) clearly established that UbiA is a *bona fide*
5-phospho-α-D-ribose-1-diphosphate: decaprenyl-phosphate-5-phosphoribosyltransferase; however, the scope of our current studies was to examine the consequences of arabinan biosynthesis and relationship between cell wall arabinan biosynthesis and DPPR/DPA precursors in a panel of mutants rather than characterization of an enzyme that was overexpressed within an Escherichia coli membrane fraction and assayed for UbiA activity.

AftA, a potential drug target, is responsible for the addition of the first key Ara residue to the galactan core, thus priming the polysaccharide for further decoration by the Emb proteins (Alderwick et al., 2005, 2006). Furthermore, UbiA represents another ideal druggable enzyme within the arabinan biosynthetic pathway, as it is responsible for the production of the only Ara sugar donor involved in mycobacterial arabinan biosynthesis and is essential for growth and survival of the organism, as discussed in Dover et al. (2004). The biosynthesis of DPA via DPPR was recently reported (Huang et al., 2005; Mikusova et al., 2005). However, it was unclear what catalytic mechanism is utilized by these enzymes; nevertheless, inspection of the UbiA sequence and further topological analysis highlights a hypothetical mechanism. Because UbiA is a membrane-bound enzyme consisting of approximately nine TM spanning domains, it would be prudent to suggest that the decaprenyl phosphate substrate is probably coordinated by these TM spanning regions anchoring the lipid acceptor in place for addition of the phosphoribose moiety from pRpp. Indeed, the most conserved region of the enzyme resides on an intracellular cytoplasmic loop, which contains the proposed catalytic motif, NDXRD, which presumably coordinates pRpp through a Mg$^{2+}$ ion allowing either $S_{N}1$ or $S_{N}2$ nucleophilic substitution.

By interruption of the gene encoding UbiA in C. glutamicum (NcgI2781), we now demonstrate that the cell wall produced from this strain is devoid of bound corynomycolic acids, deficient of arabinan, and is incapable of synthesizing DPA via DPPR. Nevertheless, arabinofuranosyltransferase activity remains unaffected by the disruption of Cg-ubiA indicating that there is no other Ara-containing donor involved in arabinan biosynthesis. Interestingly, we observed a moderate level of arabinofuranosyltransferase activity from C. glutamicumΔemb, which is attributed to a fully functional Cg-AftA (Alderwick et al., 2006), illustrating that Cg-Emb and Cg-AftA work independently from each other and thus mutually exclusively from Cg-UbiA.

Analysis of C. glutamicumΔemb and C. glutamicumΔaftA DPA biosynthetic activity resulted in two very different glycolipid profiles when the reaction products were analyzed by TLC. The reaction products were analyzed by TLC and compared with wild-type C. glutamicum. A buildup of DPPR and DPR was observed in the C. glutamicumΔemb strain, with the levels of DPA remaining consistent with wild-type C. glutamicum. This phenomenon is most likely due to a low-to-moderate turnover of DPA, which is being recruited by the first arabinosyltransferase Cg-AftA, causing a buildup of the precursors DPPR and DPR. However, C. glutamicumΔaftA produces a large reservoir of DPA, which builds up because of a complete lack of arabinofuranosyltransferase activity, both initial and downstream arabinan biosyntheses. This effect can be accredited to the fact that any endogenous cell wall...

Fig. 7. Analysis of DPA/DPR and DPPR glycolipid precursors from Corynebacterium glutamicum, C. glutamicumΔemb, C. glutamicumΔaftA, and C. glutamicum::ubiA. (A) Membranes prepared from C. glutamicum and the various mutants were assayed for DPA biosynthetic activity and analyzed via TLC and autoradiography as described in Materials and methods. (B) Individual bands corresponding to either DPPR or DPA/DPR (as numbered in panel A) were excised, hydrolyzed using 2 M trifluoroacetic acid (TFA), analyzed by TLC, and visualized by autoradiography.

1078
polysaccharide present in the membranes prepared from *C. glutamicumΔaftA* is devoid of arabinan and is therefore “unprimed” for further elongation by a fully functional Emb. Nevertheless, one would expect to observe an accumulation of DPPR and DPR in tandem with DPA buildup. Here, we propose that a critical level of DPA accumulation causes an inhibitory effect on Cg-UbiA, thus acting in a negative feedback response mechanism. Any remaining DPPR and DPR would be shuttled through the pathway via the phosphatase, Rx3790 and Rx3791 (DPR epimerase complex), respectively, which are unaffected by the level of DPA (Mikusova et al., 2005). The thermodynamics of this mechanism makes energetic sense because of the fact that a continued incorporation of pRpp with decaprenyl phosphate would be inefficient to the metabolism of the cell. Further work is required to establish the enzymatic mechanisms utilized by UbiA before potential compounds can be designed to target this essential protein in mycobacterial species.

Materials and methods

Strains and culture conditions

*Corynebacterium glutamicum* ATCC 13032 (the wild-type strain, and referred for the remainder of the text as *C. glutamicum*) and *E. coli DH5α* were grown in Luria–Bertani (LB) broth (Difco, Detroit, MI) at 30 and 37°C, respectively. The *C. glutamicum:ubiA* mutant generated in this study was grown on complex medium brain heart infusion (Difco). Kanamycin and ampicillin were used at a concentration of 50 μg/mL. Samples for lipid analyses were prepared by harvesting cells at an optical density (OD) of A600: 10–15, followed by a saline wash and freeze drying. Cultivation of *C. glutamicumΔemb* and *C. glutamicumΔaftA* were carried out as described in Alderwick et al. (2005, 2006).

Construction of plasmids

The vector used for inactivation of *C. glutamicum:ubiA* is as follows: pCG::ubiA (NCgl2781, Rx3806c). For inactivation of *ubiA*, an internal fragment of 321 bp was amplified (pubiA-for: ATC TTC AAC CAG CGC ACG ATC; pubiA-rev: AAT ATC GAT CAC TGG CAT GTG C), which was made blunt and ligated into the SmaI site of the nonreplicative vector pK18mob to yield pCG::ubiA. To enable chromosomal inactivation of *ubiA*, pCG::ubiA was introduced into *C. glutamicum* by electroporation. Selection for resistance to kanamycin yielded clones whose correct disruption of Cg-*ubiA* was confirmed with different primer pairs annealing in the vector and the bacterial chromosome.

Extraction and analysis of cell-wall-bound mycolic acids from *C. glutamicum* strains

Cells were grown as described in Strains and culture conditions, harvested, washed and freeze-dried. Cells (100 mg) were extracted by two consecutive extractions with 2 mL of CHCl3/CH3OH/H2O (10:10:3, v/v/v) for 3 h at 50°C. The bound lipids from the delipidated extracts or purified cell walls (see Isolation of the mAGP complex) were released by the addition of 2 mL of 5% aqueous solution of tetra-butyl ammonium hydroxide (TBAH), followed by overnight incubation at 100°C. After cooling, water (2 mL), CH2Cl2 (4 mL), and CH3I (500 μL) were added and mixed thoroughly for 30 min. The lower organic phase was recovered following centrifugation and washed three times with water (4 mL), dried, and resuspended in diethyl ether (4 mL). After centrifugation, the clear supernatant was again dried and resuspended in CH2Cl2 (100 μL). An aliquot (10 μL) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck, Darmstadt, Germany) and developed in petroleum ether/acetone (95:5, v/v) and charred using 5% molybdophosphoric acid in ethanol at 100°C to reveal corynomycolic acid methyl esters (CMAMEs) and compared with known standards (Gande et al., 2004).

Isolation of the mAGP complex

The thawed bacterial cells were resuspended in phosphate buffered saline (PBS) containing 2% Triton X-100 (pH 7.2), disrupted by sonication and centrifuged at 27,000 × g (Daffe et al., 1990; Besra et al., 1995). The pelleted material was extracted three times with 2% sodium dodecyl sulphate (SDS) in PBS at 95°C for 1 h to remove associated proteins, successively washed with water, 80% (v/v) acetone in water, and acetone, and finally lyophilized to yield highly purified cell wall preparations (Daffe et al., 1990; Besra et al., 1995).

Glycosyl linkage analysis of cell walls

Chemical derivitization of highly purified cell walls was carried out as described in Besra et al. (1995) and Alderwick et al. (2005, 2006). Briefly, cell wall preparations (10 mg) were suspended in 0.5 mL of dimethyl sulfoxide (anhydrous) and 100 μL of 4.8 M dimethyl sulfinyl carbamion (Daffe et al., 1990; Besra et al., 1995). The reaction mixture was stirred for 1 h, and then, CH3I (100 μL) was slowly added and the suspension stirred for a further 1 h, and this process was repeated for a total of three times. After dialysis and Sep-Pak purification, the resulting per-O-methylated cell wall samples were hydrolyzed, reduced, per-O-acetylated, and examined by gas chromatography/mass spectrometry (GC/MS) carried out on a BPX5 column (Supelco, Bellefonte, Pennsylvania) and a Finnigan Polaris/GCQ PlusTM, as described in Daffe et al. (1990) and Besra et al. (1995).

Analysis of *C. glutamicum*-free lipids

*Corynebacterium glutamicum*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA* were cultured at 30°C in 5-mL BHIS media and supplemented with antibiotic, where appropriate. Cell-wall-associated free lipids were extracted twice from 100 mg of dried cells using 2 mL of CHCl3/CH3OH/H2O (10:10:3, v/v/v) for 3 h at 50°C. Organic extracts were combined with 1.75 mL of CHCl3 and 0.75 mL of H2O, mixed, and centrifuged. The lower organic phase was recovered, back washed twice with 2 mL of CHCl3/CH3OH/H2O (3:47:48, v/v/v), dried, and resuspended with 200 μL of CHCl3/CH3OH/H2O (10:10:3, v/v/v). A 10 μL of aliquot was subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl3/CH3OH/H2O (60:16:2, v/v/v) and charred using 5% molybdophosphoric acid in ethanol at 100°C to reveal cell-wall-associated free lipids. Exported lipids were analyzed in a similar manner. *C. glutamicum*, *C. glutamicumΔaftA*,
C. glutamicumΔemb, and C. glutamicumΔaftA were cultured as described in *Strains and culture conditions*. Once the A$_{600}$ reached ~0.5, cultures were labeled with 1 μCi [14C]-acetate and further incubated for 8 h. Cells were harvested by centrifugation at 27,000 × g for 30 min, and the supernatant was carefully removed and dried using a Savant SpeedVac. The supernatant was dried and extracted using the method described in *Extraction and analysis of cell wall bound mycolic acids from C. glutamicum strains*. An aliquot of each extraction was subjected to scintillation counting and analyzed by TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O (60:16:2, v/v/v). TLCs were exposed to X-ray film (Kodak X-Omat) for 2 days to visualize radioactively labeled lipids by autoradiography.

**Preparation of corynebacterial membranes and cell wall material**

Cells (10 g) from *C. glutamicum, C. glutamicum::ubiA, C. glutamicumΔemb*, and *C. glutamicumΔaftA* were resuspended in 35 mL of 50 mM MOPS (pH 7.9), 10 mM MgSO$_4$, 5 mM β-mercaptoethanol (buffer A) and subjected to sonication for 60 s on and 90 s off (repeated for a total of 10 cycles). The cell slurry was centrifuged at 27,000 × g for 20 min at 4°C; the pellet was recovered and the resulting supernatant further centrifuged at 100,000 × g for 90 min at 4°C. Purified *C. glutamicum* membranes were recovered and resuspended in buffer A to a final concentration of 15–20 mg/mL. The pellet from the 27,000 × g spin was resuspended in 24 mL of buffer A and 32 mL of Percoll, mixed thoroughly, and centrifuged at 27,000 × g for 60 min at 4°C. The upper band (corresponding to *C. glutamicum* cell wall “P60” material) was removed, washed with buffer A with further centrifugation to remove Percoll, and the final cell wall fraction resuspended in buffer A to a final concentration of 8–10 mg/mL.

**Arabinofuranosyltransferase activity**

Membranes prepared from *C. glutamicum, C. glutamicum::ubiA, and C. glutamicumΔemb* were assayed for their ability to incorporate [14C]-Ara into endogenous cell wall polymer using either p[14C]Rpp or DP[14C]A as substrate. p[14C]Rpp and DP[14C]A were prepared, as described in Lee *et al.* (1995, 1998) and Scherman *et al.* (1996). Assays consisted of 2-mg cell membranes, 2-mg cell wall P60, 1 mM ATP, and 1 mM NADP in a final volume of 200 μL buffer A. Assays were initiated by the addition of 45,000 cpm p[14C]Rpp and incubated at 37°C for 2 h. The reaction was quenched with 6 mL of CHCl$_3$/CH$_3$OH (2:1, v/v), mixed for 15 min, and centrifuged at 27,000 × g for 15 min. The pellet was recovered, resuspended in 4 mL of CHCl$_3$/NaCl (1:1, v/v), sonicated for 5 min, and centrifuged at 27,000 × g for 15 min. This process was repeated thrice. The pellet was recovered, washed three times with 3 mL of CH$_3$OH/H$_2$O (1:1, v/v) and three times with 4 mL of CH$_3$OH, and finally dried under nitrogen before being resuspended in 5 mL of EcoSint and subjected to scintillation counting. Assays utilizing DP[14C]A as a substrate were carried out as above but with the following modifications. DP[14C]A (100,000 cpm [45 μM] prepared as described in Lee *et al.*, 1998 and stored in CHCl$_3$/CH$_3$OH [2:1, v/v]) was dried under a stream of argon in a microcentrifuge tube (1.5 mL) and placed in a vacuum desiccator for 15 min to remove any residual solvent. The dried DP[14C]A was then resuspended in 30 μL of buffer A supplemented with 10% IgePal CA-630 (Sigma Aldrich, St. Louis, MO). An aliquot of this DP[14C]A solution (40,000 cpm, 18 μM, 12 μL) was added to the remaining constituents of the assay, which were initiated by addition of the final components, incubated, and processed as described in *Arabinofuranosyltransferase activity*.

**DPA biosynthetic activity**

Cell membranes from *C. glutamicum, C. glutamicum::ubiA, C. glutamicumΔemb*, and *C. glutamicumΔaftA* were assayed for DPA biosynthesis activity. Decaprenyl phosphate (50 μg, 5 mg/mL stored in ethanol, 10 μL) was dried under nitrogen and was resuspended by the addition of 50 μL of a 1% IgePal CA-630 (Sigma Aldrich) solution in buffer A and sonicated. The basic assay mix consisted of 2 mg of membranes, 1 mM ATP, and 1 mM NADP in a final volume of 160 μL of buffer A and initiated by the addition of 45,000 cpm p[14C]Rpp. Reactions were incubated at 37°C for 2 h and quenched by the addition of 3 mL of CHCl$_3$/CH$_3$OH (2:1, v/v), mixed for 15 min, and centrifuged at 3000 × g for 15 min. The supernatant was removed, combined with 340 μL of buffer A, mixed for 15 min, and centrifuged at 3000 × g for 15 min. The lower organic phase was removed and washed twice with 1 mL of CHCl$_3$/CH$_3$OH/H$_2$O (3:47:48, v/v/v), centrifuged at 3000 × g for 15 min, recovered, and dried under nitrogen. The resulting residue was resuspended in 20 μL of CHCl$_3$/CH$_3$OH (2:1, v/v) and an aliquot subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O/MeOH (65:25:3.6:0.5, v/v/v/v) and visualized by autoradiography by exposure of TLCs to X-ray film (Kodak X-Omat). Major bands corresponding to lipid-linked sugars were excised directly from their migrating position and extracted by incubation overnight in 5 mL of CHCl$_3$/CH$_3$OH (2:1, v/v). Samples were then centrifuged at 5000 × g to remove silica gel, and the supernatant was recovered and dried under nitrogen. The products were then hydrolyzed by using 100 μL of 2 M trifluoroacetic acid (TFA) for 1 h at 120°C, dried, resuspended in 2 mL of CHCl$_3$/H$_2$O (1:1, v/v), and the upper aqueous phase recovered and dried. The residual radioactively labeled sugars were resuspended in 20 μL of H$_2$O and subjected to TLC using cellulose-coated aluminum plates (HPTLC-Aluminum Cellulose, Merck) and developed three times in formic acid/H$_2$O/tert-butanol/methyl ethyl ketone (3:3:8:6, v/v/v/v). Sugars were visualized by TLC exposure to X-ray film (Kodak X-Omat) and compared with known sugar standards.

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Conflict of interest statement

None declared.

Abbreviations

AG, arabinogalactan; Ara, arabinose; CMAME, corynomycolic acid methyl ester; DPA, decaprenylmonophosphoryl-D-arabinose; DPPR, decaprenylphosphoryl-5-phosphoribose; DPR, decapenylmonophosphoryl-D-ribose; EMB, ethambutol; Gal, galactose; mAGP, mycolyl-arabinogalactan peptidoglycan; pRpp, 5-phospho-ribofuranose-pyrophosphate; pRpp, 5-phospho-ribofuranose-pyrophosphate; TDCM, trehalose dicorynomycolates; TLC, thin layer chromatography; TM, transmembrane; TMC, trehalose monocorynomycolates.

References


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